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THE EFFECTS OF β1,3-GLUCANS ON BLOOD COAGULATION AND AMEBOCYTE RELEASE IN THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS*

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Abstract

The best-studied defense reaction in the horseshoe crab, *Limulus polyphemus,* involves the exocytotic release of a clotting system from the blood cells (amebocytes) and the formation of an extracellular clot in response to bacterial lipopolysaccharide. Although β1,3-glucans, apparently serving as signals for the presence of potentially pathogenic fungi, trigger release, blood coagulation, and activation of the prophenoloxidase system in many arthropods, *Limulus* appears to be unresponsive to endotoxin-free preparations of naturally occurring β1,3-glucans. However, the carboxymethylated β1,3-glucan derivative CMPS induced gelation of amebocyte lysates. The induction of clotting enzyme activity and gelation of amebocyte lysate by CMPS followed a complex pattern. Enzymatic activity generated by CMPS was only 15–42% of that produced by bacterial lipopolysaccharide. Neither native β1,3-glucans nor CMPS induced exocytosis of living *Limulus* amebocytes. We conclude that although CMPS produced coagulation of amebocyte lysates, the response is of questionable biological significance since native β1,3-glucans were inactive. The blood of *Limulus* lacked detectable phenoloxidase activity.

Introduction

Arthropods possess a variety of defense reactions to potentially pathogenic bacteria and fungi that gain access to the internal milieu through wounds or by active invasion through the integument (Salt, 1970; Lackie, 1980; Ratcliffe *et al.,* 1982). Many of the well studied systems involve activities of the blood cells (Bang, 1967; Levin, 1967, 1976; Ratcliffe *et al.,* 1982; Armstrong, 1985). These include phagocytosis and release of various antimicrobial systems by the exocytosis of cytoplasmic granules (Armstrong and Levin, 1979; Nachum, 1979; Nachum *et al.,* 1979; Armstrong and Rickles, 1982). The prophenoloxidase activating system appears to be an important representative of the latter category in many arthropods (Söderhäll, 1982; Söderhäll and Smith, 1984). In *Limulus,* exocytosis results in the release from the blood cells (amebocytes) of a clotting system consisting of a clottable protein (coagulogen) and a system of proteinases (Levin and Bang, 1968; Young *et al.,* 1972). The ultimate member of the proteinase cascade system (clotting enzyme) converts coagulogen, *via* a limited proteolytic cleavage, into a protein capable of polymerizing into a gel (Young *et al.,* 1972; Nakamura and Levin, 1982a, b; Torano *et al.,* 1984). This presumably functions in defense by sealing defects in the circulatory system and immobilizing microbes, thus preventing their systemic dissemination throughout the circulation (Levin and Bang, 1964a, b).

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Elements of the cell walls of pathogenic organisms appear to function as signals to host defense systems that microbes are present. Bacterial lipopolysaccharide (LPS, endotoxin) serves as an extracellular signal for the presence of gram-negative bacteria and β1,3-glucans serve as a signal for the presence of fungi. LPS is a potent activator of defense processes in *Limulus:* the blood cells can be stimulated to undergo exocytosis by LPS (Levin and Bang, 1964a; Armstrong and Rickles, 1982) and the system of proteinases involved in clotting of the coagulogen released from amebocytes also is activated by LPS (Levin and Bang, 1968; Young *et al.*, 1972; Levin, 1979). A variety of crustaceans demonstrate activation of phagocytosis, exocytosis, and the prophenoloxidase system both by LPS and by β1,3-glucans (Söderhäll and Unestam, 1979; Söderhäll, 1982, 1983; Smith and Söderhäll, 1983a, b; Söderhäll and Häll, 1984). Of interest to the present study is the possible role played by β1,3-glucans in defense reactions of blood cells of *Limulus polyphemus,* the horseshoe crab.

Materials and Methods

Limulus *amebocyte lysate*

Amebocyte lysates from the horseshoe crab, *Limulus polyphemus,* were prepared using previously described methods (Levin and Bang, 1968; Young *et al.*, 1972).

Cancer *hemocyte lysate*

Hemocyte lysates from *Cancer borealis* were prepared by withdrawing approximately 5 ml of hemolymph from individual crabs into 10 ml syringes that contained 2.5 ml of ice-cold anticoagulant (0.45 *M* NaCl; 0.1 *M* glucose; 30 m*M* trisodium citrate; 26 m*M* citric acid; and 10 m*M* Na-EDTA; at pH 4.6) (Söderhäll and Smith, 1983). The sample was centrifuged at 800 $\times g$ for 10 min, and the pellet washed once with 10 ml of anticoagulant. The hemocyte pellet was then resuspended in 1 ml of 0.01 *M* sodium cacodylate buffer, pH 7.0, homogenized, and spun at 2000 $\times g$ for 20 min to remove cell debris. The resulting supernatant was used as a source of the prophenoloxidase activating system (Söderhäll, 1982).

Astacus *hemocyte lysate*

Hemocyte lysates from the crayfish, *Astacus astacus,* were made as described by Söderhäll (1983).

Glycans

The following glycans were obtained from commercial suppliers: laminarin, a crude β1,3-glucan (Calbiochem Co., La Jolla, California); zymosan (yeast cell walls, Sigma Chemical Co., St. Louis, Missouri); hyaluronic acid (Sigma); dextran T10, T20, T40, T70, and T500 (Pharmacia, Uppsala, Sweden); cellulose (Kebo, Stockholm, Sweden); starch (Merck, Darmstadt, West Germany); and mannitol and glucose (Merck). Chitin was prepared according to Sneath (1960). Laminarin pentaose, laminarin G, and laminarin M were purified and their chemical structure elucidated as reported by Söderhäll and Unestam (1979). All glycans were dissolved or suspended in pyrogen-free distilled water.

A carboxymethylated β1,3-glucan (CPMS) with a degree of polymerization of about 540 and a degree of substitution of 0.68 was a generous gift from Dr. A. Kakinuma, Takeda Chemical Industries, Ltd., Osaka, Japan, and Dr. S. Iwanaga, Kyushu University, Fukuoka, Japan.

Endotoxin assay

Endotoxin contamination of buffers or solutions was examined using the gelation reaction of *Limulus* amebocyte lysate, as previously described (Levin and Bang, 1968). Endotoxin (lipopolysaccharide B) from *E. coli* 026:B6 was used as a standard (Difco Laboratories, Detroit, Michigan).

Assay of clotting enzyme activity in Limulus *amebocyte lysate*

Clotting enzyme activity was assayed either by the time required to form a gel at 37°C (Levin and Bang, 1968) or by measuring the generation of hydrolyzing activity toward the chromogenic synthetic peptide, Ac-Ile-Glu-Gly-Arg-pNA HCl (S-2423, AB Kabi Vitrum, Stockholm, Sweden). Clotting enzyme hydrolyzing activity, induced by different potential activators, was measured by incubating 50 μl of amebocyte lysate, 50 μl of activator, 400 μl of 0.05 *M* Tris-HCl buffer in 0.45 *M* NaCl (pH 8.0), and 100 μl of 2 m*M* S-2423 at 37°C (usually for 30 min). To stop the reaction, 100 μl of 50% acetic acid was added. The absorbance at 405 nm was measured, and enzyme activity generated in the reaction mixture is expressed as absorbance per min of incubation (A_{405}/min) (Nakamura and Levin, 1982a).

Clearly it is important to remove or inactivate endotoxin from the β1,3-glucans when testing their ability to activate the *Limulus* clotting system, since the clotting reaction is so sensitive to endotoxin that low levels of contaminating endotoxin can cause clotting even when the reagent being investigated is itself inactive (Cutler *et al.*, 1972; Rickles *et al.*, 1977, 1979; Zuckerman *et al.*, 1979). In the present study, this was accomplished by the inclusion of polymyxin B, which binds and inactivates endotoxin (Cooperstock and Riegle, 1981; Duff and Atkins, 1982; Nakamura and Levin, 1982a), and by fractionation of laminarin into the M and G chains (Söderhäll and Unestam, 1979).

Preparation of crude clotting enzyme and coagulogen

A preparation of crude clotting enzyme was generated by incubating *Limulus* amebocyte lysate with *E. coli* endotoxin (final concentration, 1 μg/ml) for 4 h at 37°C, followed by 20 h incubation at 4°C. The solid gel that formed as the result of the reaction was removed by centrifugation at 27,500 × *g* for 20 min at 4°C in a Sorvall 2-B refrigerated centrifuge. The enzyme preparation has been demonstrated previously to lack coagulogen (Young *et al.*, 1972) and is capable of gelling coagulogen (the clottable protein) present in amebocyte lysate.

A preparation of coagulogen was obtained by heating *Limulus* amebocyte lysate at 65°C for 20 min in a water bath, as described previously (Young *et al.*, 1972). The flocculent material that was produced was removed by centrifugation for 15 min at approximately 2000 × *g*. The coagulogen preparation lacked detectable clotting enzyme activity since addition of endotoxin to the coagulogen failed to produce gelation. However, incubation of the preparations of clotting enzyme and coagulogen produced a solid gel. The reaction between these preparations was not endotoxin-dependent, because it was not blocked by polymyxin B (at concentrations as high as 66,000 U/ml), a known inhibitor of bacterial endotoxins (Duff and Atkins, 1982).

Assay of phenoloxidase activity

Phenoloxidase activity in *Cancer* hemocyte lysates or *Limulus* amebocyte lysates was assayed by preincubating 100 μl of lysate with 100 μl of activator (β1,3-glucans)

for 10 min at 20°C. To this reaction mixture was then added 100 μl $MgCl_2$ (50 mM); 50 μl $CaCl_2$ (10 mM); 100 μl 0.1 M sodium cacodylate buffer (pH 7.0), and 100 μl L-dopa (1 g/l) (dihydroxyphenyl-alanine; Sigma Chemical Co., St. Louis, Missouri). These conditions are optimal for phenoloxidase activity in several marine crustaceans (Smith and Söderhäll, 1983a, b; Söderhäll and Smith, 1983). Appropriate controls always were concomitantly performed, in which pyrogen-free distilled water (Cutter Laboratories Inc., Berkeley, California) was substituted for the activator. The enzymatic reaction was allowed to proceed for different lengths of time at 20°C and enzyme activity is expressed as absorbance at 490 nm/minute.

Assay of protease activity in crayfish hemocyte lysate

The protease activity of hemocyte lysate supernatants (70,000 × g, 20 min) was assayed using the chromogenic peptide, Bz-Ile-Glu-(γ-O-piperidyl)-Gly-Arg-pNA (S-2337, AB Kabi Vitrum, Molndal, Sweden) as substrate, as described by Söderhäll (1983).

Miscellaneous

Other chemicals used were: polymyxin B (Burroughs Wellcome Co., Triangle Park, North Carolina) and L-dihydroxyphenyl-alanine (Sigma). All other chemicals were of analytical grade. All glassware was rendered sterile by autoclaving at 150°C for 45 min and then made pyrogen-free by heating in a dry oven at 190°C for 4 h.

Exocytosis

Exocytosis was studied by direct microscopic observation of amebocytes adherent to microscope coverglasses mounted in a simple perfusion chamber, as described by Armstrong and Rickles (1982). Five to ten drops of blood were diluted in 15 ml of sterile, endotoxin-free 3% NaCl, contained in an endotoxin-free 90 mm petri dish with 4–6 22 mm coverglasses on the bottom. The cells settle and attach to the coverglasses in 1–5 min, which can then be transferred to a perfusion chamber constructed by inverting a coverglass over a drop of saline on a sterile, endotoxin-free microscope slide. The coverglass is supported above the slide on fragments of a No. 1½ coverglass. The cells can then be presented with various agents dissolved in 3% NaCl that is perfused beneath the coverglass. The progress of degranulation was ascertained by periodic microscopic examination (Armstrong and Rickles, 1982).

RESULTS

Induction of clotting enzyme activity by natural glycans

Several glycans were tested for their ability to induce clotting enzyme activity in *Limulus* amebocyte lysate, using the gelation assay (see Materials and Methods) or by recording hydrolysis of the synthetic peptide, Ac-Ile-Glu-Gly-Arg-pNA (S-2423). Of the natural glycans tested, only a crude β1,3-glucan (laminarin) induced significant clotting enzyme activity in amebocyte lysate. Zymosan, a yeast cell wall preparation, only slightly affected the system, and all other natural glycans tested were negative (Table I). The optimal concentrations of laminarin for activation were greater than 0.01 mg/ml.

Interestingly, two purified subfractions of laminarin, laminarin G (Lam G) and laminarin M (Lam M) (Söderhäll and Unestam, 1979), were inactive. The former is

TABLE I

Effects of different glycans on clotting enzyme activity of Limulus *amebocyte lysate*

Glycan	Optimal concentration (mg/ml)	Structure	Amidase activity (ΔA_{405} nm/min/ml reaction mixture)
Laminarin	10^{-1}	(1–3) β-D-glucan	0.14
CMPS[1]	10^{-4}	(1–3) β-D-glucan, carboxymethylated	0.032
Zymosan	10^{-4}	yeast cell walls	0.006
Laminarin G	Not active[3]	(1–3) β-D-glucan terminated with glucose	0
Laminarin M	Not active[3]	(1–3) β-D-glucan terminated with mannitol	0
Laminaripentaose	Not active[3]	(1–3) β-D-glucan	0
Dextran T10, T20, T40, T70, T500	Not active[3]	α-D-glucan	0
Hyaluronic Acid	Not active[3]	(1–4) β-D-glucuronic acid; (1–3) β-N-acetyl-D-glucosamine	0
Cellulose	Not active[3]	(1–4) β-D-glucan	0
Starch	Not active[3]	α-D-glucan	0
Chitin[2]	Not active[3]	(1–4) β-N-acetyl-glucosamine	0
Cellobiose	Not active[3]	(1–4) β-D-glucan	0
Glucose	Not active[3]	—	0
Mannitol	Not active[3]	—	0
Endotoxin	10^{-3} to 10^{-4}	—	0.14

[1] Provided by Dr. A. Kakinuma.
[2] Tested as a suspension.
[3] All glycans were tested at concentrations ranging from 10^{-8}–10^{-1} mg/ml.

terminated with glucose and the latter with mannitol. Neither laminarin G nor M, in concentrations ranging from 10^{-8} to 1 mg/ml, activated the clotting system of *Limulus* amebocyte lysate (Table I). A small linear pentasaccharide (laminaripentaose), composed of (1–3) linked β-D-gluco-pyranosyl residues, also was ineffective in activating the proclotting enzyme (Table I).

We investigated the possibility that the activity of crude laminarin was due to a LPS contaminant. Laminarin lost all its activating capacity after being heated for 2 h at 180°C, a treatment which destroys the activity of LPS in the *Limulus* clotting system (Atkins and Heijn, 1965). More specifically, polymyxin B, which by binding to LPS (Cooperstock and Riegle, 1981) inhibits LPS-activation of proclotting enzyme activity (Nakamura and Levin, 1982a), also blocked activation by laminarin (Fig. 1). The β1,3-glucans of the laminarin preparation were unaffected by the polymyxin B treatment since we detected no significant differences in amidase or prophenoloxidase activation in hemocyte lysate from decapod crustaceans between polymyxin B-treated and untreated laminarin (Table II). Thus, we concluded that native glucans are unable to activate the *Limulus* clotting system, provided that they are free of endotoxin contamination.

Induction of clotting enzyme activity by the carboxymethylated glucan, CMPS

It has been reported that a derivatized glucan is capable of activating the clotting enzyme in lysates of amebocytes of the Japanese horseshoe crab, *Tachypleus tridentatus* (Kakinuma *et al.*, 1981; Morita *et al.*, 1981). The β1,3-glucan used by Kakinuma *et al.* (1981) and Morita *et al.* (1981) to induce activation of the horseshoe crab clotting

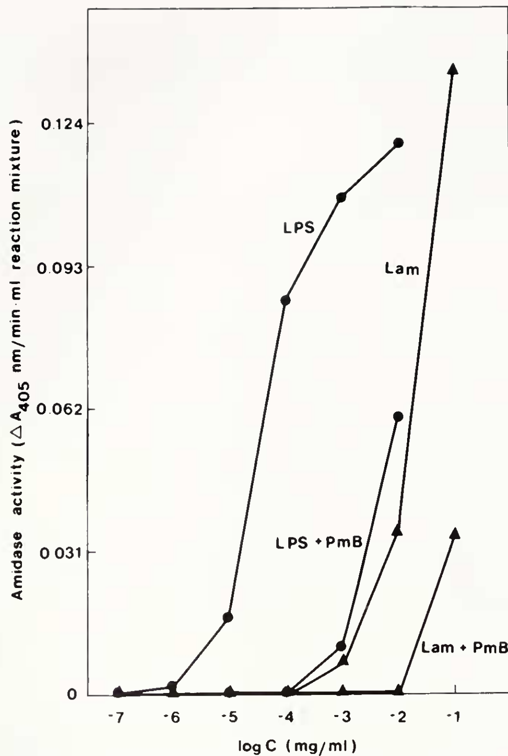

FIGURE 1. Effects of polymyxin B treatment of lipopolysaccharide (LPS) or laminarin (Lam) upon induction of clotting enzyme activity in *Limulus* amebocyte lysate. Various concentrations (C) of LPS or Lam were pretreated with polymyxin B (PmB) (100 U/ml) for 30 min before they were assayed for the induction of clotting enzyme activity, using the chromogenic peptide assay (see Materials and Methods).

system is a carboxymethylated curdlan (CMPS), with a degree of polymerization of approximately 500. In our hands, this CMPS also activated proclotting enzyme and generated clotting enzyme activity (Table I), which resulted in subsequent gelation of *Limulus* amebocyte lysate. Polymyxin B (final concentration, 1000 U/ml) did not affect CMPS-induced activation of proclotting enzyme activity, suggesting that activation was not due to endotoxin contamination (data not shown). Activation showed a curious dependence upon dosage, with inhibition occurring at CMPS concentrations above 10^{-2} mg/ml (data not shown) as also has been reported by Kakinuma *et al.* (1981). At these high concentrations (greater than 10^{-2} mg/ml), the CMPS preparation caused the immediate formation of a precipitate in *Limulus* lysate which could not be redissolved in 1% NaCl or distilled water. The supernatant prepared by removing this precipitate still contained endotoxin-inducible hydrolyzing activity towards Ac-Ile-Glu-Gly-Arg-pNA and coagulogen (Table III). This indicates that high concentrations of CMPS did not inactivate or precipitate critical components in the "endotoxin pathway" of the *Limulus* lysate clotting system or precipitate the coagulogen. Thus, based on these observations, it is suggested that there are two separate pathways in the clotting system of Limulidae, one activated by endotoxin and the other by CMPS. Consistent with this suggestion, different lysate preparations exhibited different sensitivities for CMPS, which did not correlate with their sensitivities to endotoxin (Table

TABLE II

Effect of polymyxin B treatment of a β1,3-glucan (laminarin G) upon activation of an endogenous proteinase and prophenoloxidase in decapod hemocyte lysates

Treatment of hemocyte lysate			
β1,3-glucan (mg/ml)	Polymyxin B (units/ml)	Amidase activity[1] (ΔA_{405} nm/min/ml)	Phenoloxidase activity[2] (ΔA_{490} nm/min/ml)
0.025	100	—	0.028
0.025	0	—	0.026
0.01	1000	0.036	—
0.01	100	0.039	—
0.01	0	0.042	—
0.0025	100	—	0.018
0.0025	0	—	0.017
0.001	1000	0.018	—
0.001	0	0.025	—
0	100	0.003	0.009
0	0	0.004	0.009

[1] Laminarin G (a β1,3-glucan) was treated with polymyxin B for 15 min at 20°C. Polymyxin B treated or untreated laminarin G then was assayed for capacity to activate serine proteases in the hemocyte lysate of the crayfish, *Astacus astacus*, by preincubating 100 μl hemocyte lysate with 100 μl treated or untreated laminarin G for 10 min at 20°C. After the preincubation, 100 μl of this mixture was added to 400 μl 0.01 *M* Tris-HCl buffer, pH 8.0 and 100 μl 1.5 m*M* chromogenic peptide (Bz-Ile-Glu-(γ-0-piperidyl)-Gly-Arg-pNA), and incubated at 37°C for 30 min. To terminate the reaction, 100 μl 50% acetic acid was added and then absorbance at 405 nm was measured.

[2] Laminarin (100 μl) was pretreated with polymyxin B (final concentration, 100 U/ml) for 2 h. It then was incubated with 100 μl of hemocyte lysate from the crab, *Cancer borealis*, and 100 μl of 250 m*M* $MgCl_2$ for 15 min, followed by incubation with 100 μl of L-dopa (3 g/l). Phenoloxidase activity was measured after 20 min incubation with L-dopa at 20°C (see Materials and Methods). Water was substituted for laminarin to ascertain that polymyxin B did not generate phenoloxidase activity.

IV). It is noteworthy that enzymatic activity generated by CMPS was only 15–42% of that produced by endotoxin (Tables I and IV).

Exocytosis of the Limulus *blood cell (amebocyte)*

In the unchallenged animal, the entire coagulation system of *Limulus* is contained within the secretory granules of the blood cells (Mürer *et al.*, 1975). The first event

TABLE III

Endotoxin activation and gelation of CMPS-inhibited Limulus *amebocyte lysate*[1]

Treatment of CMPS inhibited lysate	Gelation	Amidase activity (ΔA_{405} nm/min/ ml reaction mixture)
Endotoxin (10^{-4} mg/ml)	Yes	0.11
CMPS (10^{-2} mg/ml)	No	0

[1] CMPS (1 mg/ml) was incubated with an equal volume of amebocyte lysate for 1 h at 20°C. The mixture then was centrifuged for 20 min at 27,500 × *g* (6°C) to remove the precipitate which had formed, and the resulting supernatant tested for the ability to support production of hydrolyzing activity towards S-2423 or for the presence of clotting enzyme, following addition of endotoxin or additional CMPS. Control preparations of lysate caused to gel by the addition of 10^{-4} mg/ml endotoxin (no CMPS present) contained 0.14 units of amidase activity.

TABLE IV

Comparison of LPS or CMPS activation of different batches of Limulus *amebocyte lysate*[1]

	Amidase activity (ΔA_{405} nm/min/ml reaction mixture)		
	61–80[3]	64–80	156–81
LPS (10^{-4} mg/ml)[2]	0.136	0.098	0.139
CMPS (10^{-4} mg/ml)	0.020	0.033	0.059

[1] LPS or CMPS-generated clotting enzyme activity in different batches of *Limulus* amebocyte lysates was measured using the chromogenic peptide assay (see Materials and Methods).

[2] Concentrations of LPS and CMPS of 10^{-4} mg/ml were selected because CMPS was found to be inhibitory at higher concentrations.

[3] Numbers refer to batch number of amebocyte lysate.

necessary for coagulation to occur is the release of the enzymes and coagulogen, an event that occurs by exocytotic degranulation (Dumont *et al.*, 1966; Ornberg and Reese, 1981; Armstrong and Rickles, 1982). Degranulation of washed amebocytes maintained in observation chambers can be triggered by exposure to specific secretagogs dissolved in 3% NaCl. For example, degranulation of a monolayer of amebocytes occurred within 3–10 min following exposure to 10 μg/ml of bacterial lipopolysaccharide (LPS) (Fig. 2a, b), as observed previously by Armstrong and Rickles (1982). In this system washed amebocytes were nonresponsive to high concentrations (100 μg/ml, 30–60 min) of zymosan, CMPS (Fig. 2c), and two purified preparations of β1,3-glucans (laminarin G and M). After a 60 min exposure to the purified β1,3-glucan preparations, the cells were still capable of degranulating when exposed to LPS (10 μg/ml). Different amebocyte preparations varied in their response to laminarin; at high concentrations (100 μg/ml), some preparations degranulated whereas others did not. We interpret the positive responses that were occasionally observed with laminarin as resulting from the presence of LPS in this material (see Fig. 1). Pertinently, it has previously been demonstrated (Armstrong and Rickles, 1982) that some preparations of *Limulus* amebocytes are more sensitive than others to low doses of LPS (1 μg/ml). Apparently, the intact washed amebocyte does not recognize the β1,3-glucans as secretagogs, which is in contrast to the situation in crustaceans in which the semi-granular cells respond by exocytosis if challenged with β1,3-glucans (Johansson and Söderhäll, 1985).

Phenoloxidase activity in Limulus *blood*

The prophenoloxidase activating system of crustaceans and insects is important in the killing of invading parasites and microbes (Salt, 1970; Söderhäll, 1982) and in tanning of the cuticle after molting and during wound healing (Neville, 1975). The system can be activated by β1,3-glucans (Unestam and Söderhäll, 1977; Ashida, 1981; Ashida *et al.*, 1983; Smith and Söderhäll, 1983a; Söderhäll, 1983; Leonard *et al.*, 1985) and by bacterial products (Pye, 1974; Ashida *et al.*, 1983; Söderhäll and Häll, 1984). This enzyme system, so important in cellular defense in other arthropods, could not be detected by the standard biochemical tests in *Limulus* (Table V). Its absence also is suggested by the failure of aggregated preparations of living, extravasated amebocytes to melanize, even after maintenance for several days in organ culture (Fig. 3).

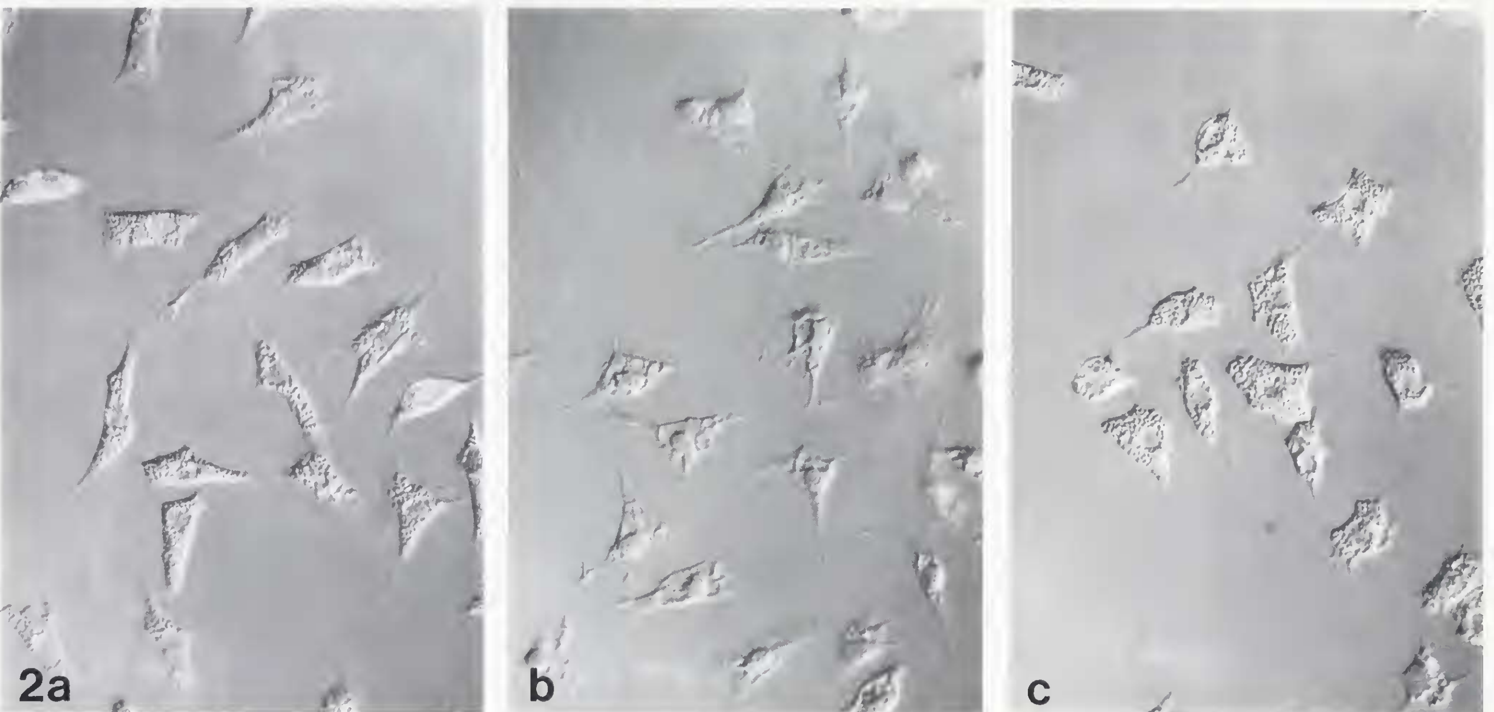

FIGURE 2. The ability of potential secretagogs to induce exocytosis of *Limulus* amebocytes can be studied by differential interference contrast microscopic examination of cells adherent to microscope coverglasses. The agent to be studied, in this case bacterial lipopolysaccharide (10 μg/ml), was dissolved in 3% NaCl and perfused beneath the coverglass. In Figure 2a, taken 1 min after exposure of the cells, the amebocytes are still fully granular (the granules are the refractile, oval bodies in the cytoplasm). Degranulation occurred at 4 min and by 5 min (Fig. 2b) the cells had completed exocytotic release of the granules. The glucan CMPS did not induce exocytosis of washed amebocytes at a concentration of 100 μg/ml. Time of exposure: 47 min (Fig. 2c). Similarly, zymosan and laminarins G and M were without effect (not shown). 470×.

TABLE V

Assays for phenoloxidase activity in Limulus *hemolymph*

Treatment of *Limulus* amebocyte lysate (LAL)	Phenoloxidase activity[1] ΔA_{490} nm/60 min ml reaction mixture
LAL	0
LAL + trypsin (0.25 mg/ml)	0
LAL + $CaCl_2$ (10 mM)	0
LAL + $CaCl_2$ (10 mM) + trypsin (0.25 mg/ml)	0
LAL + $MgCl_2$ (50 mM)	0
LAL + $MgCl_2$ (50 mM) + trypsin (0.25 mg/ml)	0
LAL + $CaCl_2$ (10 mM) + $MgCl_2$ (50 mM) + trypsin (0.25 mg/ml)	0
Limulus plasma	0
Limulus plasma + trypsin	0

[1] Enzyme activity determined as detailed in Materials and Methods.

DISCUSSION

Limulus, like many large arthropods, is relatively long-lived, requiring 9–12 years to reach maturity (Shuster, 1950, 1954), and with a maximum lifespan estimated to be in excess of 14–19 years (Ropes, 1961). One requirement for such longevity is the ability to defend against potentially pathogenic microbes (for review see Armstrong, 1985). The cuticle and epidermis certainly form important barriers to penetration. The one well-studied internal defense mechanism in *Limulus* is activation of the clotting system by bacterial endotoxin (Bang, 1956, 1979; Shirodkar *et al.,* 1960; Levin and Bang, 1964a, 1968; Levin, 1967; Stagner and Redmond, 1975). Other arthropods

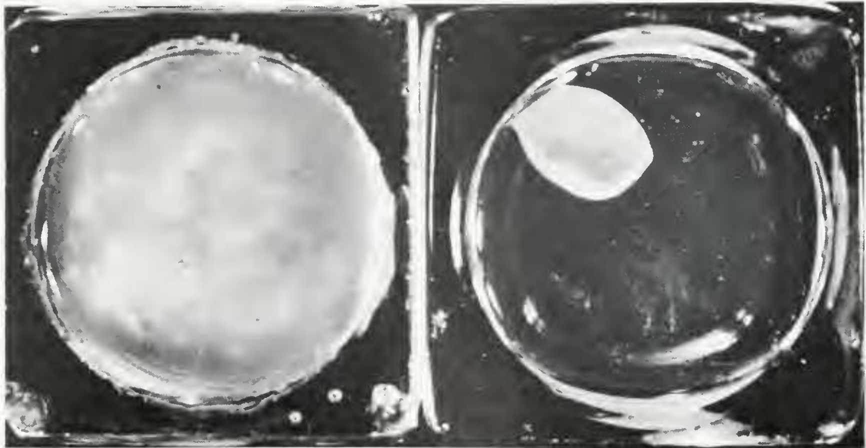

FIGURE 3. Amebocyte tissue can be prepared for study by collecting blood under aseptic conditions in embryo watchglasses. After removal from the animal, the blood cells settle and aggregate into a tissue-like mass that, after an extended period *in vitro,* undergoes contraction. In right-hand watchglass, it is 1 day old. The amebocyte tissue mass has contracted into the compact, white, button-like mass in the upper left of the watchglass. Even at 1 or more days after preparation, the aggregated mass of blood cells is white in color, with no sign of melanization. The fluid medium was *Limulus* plasma.

also respond to endotoxin by degranulation of certain classes of granular blood cells (Johansson and Söderhäll, 1985) and activation of the prophenoloxidase activating system (Söderhäll and Häll, 1984). In both crustaceans and *Limulus,* endotoxin apparently serves as an important humoral signal for the activation of appropriate host defense systems in the presence of gram-negative bacteria.

Fungi represent another important class of microbial pathogens for arthropods (Johnson, 1970; Sindermann, 1970). The β1,3-glucans, which are components of the cell walls of all fungi except the mucorales (Bartnickii-Garcia, 1972), serve as humoral activators of the defense systems of crustacean (Unestam and Söderhäll, 1977; Söderhäll, 1981; Smith and Söderhäll, 1983a, b) and insect (Pye, 1974; Ashida *et al.,* 1983; Leonard *et al.,* 1985) blood cells. These compounds, like endotoxin, stimulate blood cell clumping (Smith *et al.,* 1984) and exocytosis (Smith and Söderhäll, 1983b) and activation of the prophenoloxidase system (Söderhäll and Unestam, 1979; Ashida, 1981; Söderhäll, 1982; Ashida and Söderhäll, 1984).

The purpose of the present study has been to investigate the possibility that similar recognition-response patterns to fungal cell wall components are displayed by the blood cells of *Limulus,* as a representative of the chelicerate arthropods. Our results indicate that whole fungal cell walls (zymosan) and the naturally occurring β1,3-glucans are not recognized by the clotting system of *Limulus,* confirming previous reports that extracts from a variety of yeasts fail to gel *Limulus* amebocyte lysate (Cutler *et al.,* 1972; Jorgensen and Smith, 1973; Wildfeuer *et al.,* 1975). Although the artificially derivatized β1,3-glucan, CMPS, did trigger coagulation of the isolated clotting system, endotoxin-free preparations of naturally occurring β1,3-glucans were inactive. The production of a precipitate following exposure of *Limulus* amebocyte lysates to high concentrations of CMPS, which did not occur with any of the other tested glucans, suggests that the reaction between CMPS and horseshoe crab lysate may be complex. The observation that the maximal enzymatic activity generated by CMPS was only 15–42% of that produced by LPS is difficult to rationalize with the notion that both activate the same enzyme cascade system. In sum, the biological significance and underlying mechanisms of activation of the clotting system by CMPS are unclear. In addition, CMPS, zymosan, and all of the endotoxin-free natural β1,3-glucan preparations that were tested did not provoke exocytosis of isolated *Limulus* blood cells. Since, in the unchallenged animal the entire clotting system is contained within the exocytotic granules of the blood cells, and, under natural conditions, must be released by exocytosis to become active (Mürer *et al.,* 1975), this latter observation suggests that any naturally occurring β1,3-glucans that might be structural and functional homologues to CMPS would be unlikely to play a functional role in defense because CMPS does not trigger the release reaction.

A second interesting observation made during the present study is that *Limulus* blood apparently lacks prophenoloxidase activity. This system is widely distributed in the arthropods and serves several important roles in defense (Ashida, 1971, 1981; Söderhäll, 1982; Ashida *et al.,* 1983; Ashida and Söderhäll, 1984; Söderhäll and Häll, 1984; Leonard *et al.,* 1985). The biochemical assay conventionally used to assay phenoloxidase activity in the blood of insects and crustaceans consistently failed to reveal activity in the blood or lysates of the blood cells of *Limulus.* Also, isolated blood cells failed to melanize following bleeding, consistent with previous observations that melanization does not occur during wound healing in *Limulus* (Bursey, 1977).

In summary, the blood-based defense systems of *Limulus* appear to lack certain characteristics seen in insects and crustaceans, namely the ability to respond to fungi or fungal cell wall glucans and the ability to activate phenoloxidase in response to bacterial or fungal cell wall products. It will be of interest to discover whether these

deficits are present in other chelicerate arthropods. It also will be important to elucidate the mechanisms that confer resistance of *Limulus* to potentially pathogenic fungi.

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